REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

Claim Status and Amendments

Claims 12, 13 and 15-23 were pending in this application when last examined.

Claims 17-22 were withdrawn as non-elected subject matter.

Claims 12-13, 15-16 and 23 were examined on the merits and stand rejected.

Claim 12 is amended. Support can be found on page 5, lines 8-10, page 9, line 18 and in the Example. In particular, Applicants note that such amendment is not new matter as subject matter which a person of skill in the art would reasonably conclude that Applicants had possession of at the time of filing is not new matter.

Written Description Rejection

On pages 3-5, claims 12-13, 15-16 and 23 were rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Applicants respectfully traverse this rejection as applied to the amended claims.

It is obvious based upon the above passages with regard to supporting the claim amendments, in particular the double stranded DNA of the Example, that Applicants had possession of target DNA which is double-stranded and has a sense strand which encodes a protein. Applicants further note the Examiner's own definition wherein the term "sense" strand is usually defined with respected to double-stranded DNA and direction of protein encoding.

The Examiner is reminded that the test of possession is what a person of skill in the art would reasonably understand Applicants had possession at the time of filing. Applicants respectfully submit that they reasonably have possession of the claimed invention including wherein the target is double-stranded DNA and its sense strand encodes a protein.

The Examiner also indicates that the specification lacks support for correcting a sequence as set forth in claims 15 and 23. Claim 15 merely indicates that the target DNA sequence in the cell is a DNA sequence causing a disease due to the one or more bases. The art is replete with examples of DNA sequences with base mutations which cause disease. The Examiner has failed to set forth any rational underpinning indicating why a person of skill in the art would not think that Applicants had possession of using such DNA sequence in the claimed method of *in vitro* base conversion. The Examiner has failed to set forth any evidence that such DNA sequences

would act any different in the claimed method than other DNA sequences. Thus, with regard to claim 15, this rejection is moot.

Furthermore, claim 23 is directed towards converting genomic or mitochondrial DNA. The Examiner has failed to set forth any technical reason why mitochondrial or genomic DNA would behave any differently in the method of the claimed invention. Thus, this rejection is moot.

In view of the above, Applicants respectfully suggest that this rejection is untenable and should be withdrawn.

Enablement Rejection

On pages 5-9, claims 12-13, 15-16 and 23 were rejected under 35 U.S.C. § 112, first paragraph, for failure to meet the enablement requirement. Applicants respectfully traverse this rejection as applied to the amended claims.

Example in specification supports eukaryotic cells

In opposition to the statements of the Examiner, Applicants respectfully note that the Example in the specification shows the claimed *in vitro* base conversion method in eukaryotic cells. Such conversion is then confirmed in E. coli cells, which apparently has resulted in confusion.

In particular, the Example of this application explicitly describes that:

i) A mutated HygEGFP gene is introduced into plasmid pTriEX-3Noe (Novagen, WI) to obtain a target plasmid pTENHEX. Please note that the target plasmid is not phage plasmid.

ii) The target plasmid pTENHEX is introduced into CHO-K1 cell together with a DNA fragment, f Sense, which is a sense strand of 606nt in HygEGFP gene. Please note the CHO-K1 is derived from "Chinese hamster ovary cell", which is a mammalian cell.

iii) Replacement of base in the target double-stranded plasmid pTENHEX (gene correction) is done in CHO-K1 cell with an action of f Sense.

iv) Determination of gene correction is done by recovering the target plasmid pTENHEX from CHO-K1 cell and introducing the plasmid into E. coli cell. This method, i.e., a plasmid is recovered from mammalian cell and introduced into E. coli cells is a well known assay method (for example, Yoon et al. Proc. Natl. Acad. Sci. USA, 93:2071-2079, 1996, attached herewith).

Thus, the above example provides a reasonable basis that the claimed invention can be practiced without undue experimentation.

The **scope** of the claims is enabled

Furthermore, turning to the Examiner's reasoning on pages 6 and 7, the Examiner appears to set forth problems with *in vivo* gene repair based on the Nature Reviews Genetics article. However, Applicants note that MPEP 2164.08 specifically directs that the focus to the examination inquiry is whether everything within the scope of the claims is enabled. The Examiner appears to be instead focusing on downstream effects of converted bases in organisms or after further cell culture. This is not the proper test for the present claims. Instead, the proper test is whether the claimed invention will result in the base conversion without undue experimentation. Based on the above-noted Example, it is respectfully requested that the Applicants have shown that the enablement requirement is met. On the other hand, the Examiner has failed to make a *prima fascia* case as the Examiner has failed to limit the inquiry to the scope of the claims.

The Examiner has failed to make a *prima fascia* case

Furthermore, the Examiner is also directed towards MPEP 2164.08(b). It is noted that even if the Examiner can set forth some instances where *in vitro* base conversion by the claimed methodology would not work, such is not an indicator that the claims fail to meet the enablement requirement. As noted in this MPEP section, the standard is whether a skilled person could determine which embodiments that were conceived but not yet made, would be inoperative or operative without expenditure of no more effort than is normally required in the art. The Examiner has failed to make a *prima fascia* case that there are nonworking embodiments and such nonworking embodiments could be determined only by undue experimentation.

Thus, in view of the above, Applicants respectfully suggest that this rejection is untenable and should be withdrawn.

Anticipation Rejection

Finally, in item 11 on pages 10-11, claims 12-13, 16 and 23 were newly rejected under 35 U.S.C. § 102(b) as anticipated by Bilang et al. (1992. Molecular and Cellular Biology. Vol. 12, No. 1, pp. 329-336) as evidenced by Genbank Accession No. JA 136868. Applicants respectfully traverse this rejection as applied to the amended claims.

Applicants note that claim 12 has been amended without prejudice to recite that the target DNA is double-stranded. On the other hand, the target DNA of Bilang et al. is single-stranded. Thus, this rejection is untenable and should be withdrawn.

Furthermore, in Bilang et al., the experiment in Fig. 4 shows the efficiencies of homologous recombination between pBSKB1**ds** (double-stranded) and pTZR3**ss** (single-stranded). This may be similar to the claimed invention, since a base conversion was observed by using **ds** DNA and **ss** DNA. However, the efficiency of homologous recombination (i.e., number of hygromycin resistant colonies) is 81, 68, 150 between pBSKB1**ds** and pTZR3**ss**, and 315, 306, 233 between pBSKB1**ds** and pTZR3**ds**. This shows that the efficiency of homologous recombination is superior between **ds** DNA and **ds** DNA, compared with **ds** DNA and **ss** DNA.

Thus, there would be no articulated reason with rational underpinning to modify the invention of Bilang et al. to arrive at the claimed invention. Furthermore, the examples in the specification show the superior efficiency of single-stranded sense DNA fragment, which does not depend on other conditions. For instance, please see Figs. 3 and 4.

Thus, Applicants respectfully request the Examiner to again consider the evidence of superior conversion efficiency shown in the specification. Such superior conversion efficiency is due to use of single-stranded sense DNA fragment and therefore is inherently recited in the claims. Such superior properties are not taught or suggested in Bilang.

Thus, in view of the above, Applicants respectfully suggest that this rejection is untenable and should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Hiroyuki KAMIYA et al.

/William R. Schmidt, II/

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Genetics

Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA·DNA oligonucleotide

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Communicated by I. Tinoco, Jr., University of California, Berkeley, CA, October 11, 1995

ABSTRACT **An experimental strategy to facilitate correction of single-base mutations of episomal targets in mammalian cells has been developed. The method utilizes a chimeric oligonucleotide composed of a contiguous stretch of RNA and DNA residues in a duplex conformation with double hairpin caps on the ends. The RNA/DNA sequence is designed to align with the sequence of the mutant locus and to contain the desired nucleotide change. Activity of the chimeric molecule in targeted correction was tested in a model system in which the aim was to correct a point mutation in the gene encoding the human liver/bone/kidney alkaline phosphatase. When the chimeric molecule was introduced into cells containing the mutant gene on an extrachromosomal plasmid, correction of the point mutation was accomplished with a frequency approaching 30%. These results extend the usefulness of the oligonucleotide-based gene targeting approaches by increasing specific targeting frequency. This strategy should enable the design of antiviral agents.**

Targeted correction of disease-related mutations or site-directed inactivation of viral genes by homologous recombination would be a effective strategy for gene therapy. Unfortunately, homologous recombination in mammalian cells between a target gene and an exogenous DNA vector takes place at relatively low frequencies and is complicated by interference from an illegitimate recombination pathway that does not depend on sequence homology (1–5). An alternative approach involves targeted mutagenesis facilitated by triple-helix-forming oligonucleotides coupled to cross-linking agents (6, 7). Such oligonucleotides have been used previously to change DNA sequences thereby altering gene expression but these approaches have been limited by the sequence restriction of the target that must consist of homopurine or homopyrimidine stretches (8, 9). Moreover, the generation of a specific type of mutation or correction has been difficult to achieve (6, 7).

We have developed an experimental strategy to enable correction of single-base mutations of episomal sequences by using a chimeric oligonucleotide of unique design. This strategy evolved from *in vitro* studies on homologous recombination conducted with the RecA and Rec2 proteins (10–12). Analysis of the homologous pairing reaction promoted by the Rec2 protein of *Ustilago maydis* revealed that RNA·DNA hybrids were more active in homologous pairing reactions than corresponding DNA duplexes (10). Since pairing would appear to be the rate-limiting step during the gene targeting process (13), the overall frequency of recombination should be elevated if the number of pairing events is elevated. It was also discovered that joint molecule formation proceeded efficiently even when the ends of the hybrid were capped with double hairpin structures. These observations led us to a strategy for gene targeting in which vector design would exploit the natural recombinogenicity of RNA·DNA hybrids and would feature double-hairpin capped ends avoiding destabilization or destruction by cellular helicases or exonucleases. A chimeric oligonucleotide can be designed so that it aligns in perfect register with a specified genomic target or in imperfect register such that a single base pair is different between the oligonucleotide and a specified targeted nucleotide. In the latter case, structural distortion created by the mismatched base pair should be recognized by the endogenous repair systems (14, 15) and a change in sequence on either chimeric oligonucleotide or the target sequence would ensue. An additional feature in the design of chimeric oligonucleotides was modification of the RNA residues by 2′-O-methylation of the ribose sugar (16) to render the oligonucleotide resistant to the RNase H activity present in mammalian cells.

To test the feasibility of chimeric oligonucleotide-based targeting in mammalian cells, we chose an episomal target utilizing human liver/bone/kidney alkaline phosphatase cDNA, whose gene product is important in skeletal mineralization. Mutations in the structural gene give rise to hypophosphatasia, a metabolic bone disease with variable clinical severity ranging from still birth with almost no mineralized bone to pathological fractures in adults (17). A well-characterized mutant form has a missense mutation (G → A) at position 711 of the cDNA that results in the loss of enzymatic activity (18). This particular gene was chosen for study since direct biochemical and histochemical assays are available to monitor its activity. With the use of an appropriate chromogenic substrate dye, enzymatic activity can be detected by deposition of pigment on the cells since the enzyme localizes to the cell surface (19). Chinese hamster ovary (CHO) cells were chosen as hosts since there is little detectable endogenous alkaline phosphatase expressed (19, 20). A gene correction event mediated by chimeric oligonucleotides would restore enzymatic activity and be visualized by pigment development. Thus, targeted gene correction can be detected directly without selection or screening for the rare successful targeting events. In this paper, we demonstrate that targeted mutagenesis by chimeric oligonucleotide occurs at a high frequency in a sequence-specific manner.

MATERIALS AND METHODS

Synthesis and Purification of Oligonucleotides. The chimeric oligonucleotides were synthesized on a 0.2-μmol scale by using the 1000-Å-wide-pore CPG on the ABI 394 DNA/RNA synthesizer. The exocyclic amine groups of DNA phosphoramidites (Applied Biosystems) are protected with benzoyl for adenosine and cytidine and isobutyryl for guanosine. The 2′-O-methyl RNA phosphoramidites (Glen Research, Sterling, VA) are protected with a phenoxyacetyl group for adenosine, dimethylformamide for guanosine and an isobutyryl group for cytidine. After the synthesis was complete, the base-protecting groups were removed by heating in ethanol/concentrated ammonium hydroxide, 1:3 (vol/vol), for 20 h at 55°C. The crude oligonucleotides were purified by polyacrylamide gel

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Abbreviations: FBS, fetal calf serum; SV40, simian virus 40.

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electrophoresis. The entire oligonucleotide sample was mixed with 7 M urea/10% (vol/vol) glycerol, heated to 70°C, and loaded on a 10% polyacrylamide gel containing 7 M urea. After gel electrophoresis, DNA was visualized by UV shadowing, dissected from the gel, crushed, and eluted overnight in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.5) with shaking. The eluent containing gel pieces was centrifuged through 0.45-μm (pore size) spin filter (Millipore) and precipitated with ethanol. Samples were further desalted with a G-25 spin column (Boerhinger Mannheim) and greater than 95% of the purified oligonucleotides was found to be full length.

Transient Transfection and Measurements of Alkaline Phosphatase Activity. CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BRL) containing 10% (vol/vol) fetal bovine serum (FBS; BRL). Transient transfection was carried out by addition of 10 μg of the plasmid with 10 μg of Lipofectin in 1 ml of Optimem (BRL) to 2×10^5 CHO cells in a 6-well plate. After 6 h, various amounts of oligonucleotide was mixed with 10 μg of Lipofectin in 1 ml of Optimem and added to each well. After 18 h, the medium was aspirated and 2 ml of DMEM containing 10% FBS was added to each well. Histochemical staining was carried out (19), 24 h after transfection of the oligonucleotide. Spectrophotometric measurements were carried out by the ELISA amplification system (BRL). Transfection was carried out in triplicate in a 96-well plate. The amounts of reagents and cells were 10% of that used for the 6-well plate. Cells were washed three times with 0.15 M NaCl and lysed in 100 μl of buffer containing 10 mM NaCl, 0.5 Nonidet P-40, 3 mM $MgCl_2$, and 10 mM Tris·HCl (pH 7.5), 24 h after transfection with chimeric oligonucleotides. A fraction of cell lysates (20 μl) was incubated with 50 μl of ELISA substrate and 50 μl of ELISA amplifier (BRL), the reaction was stopped by addition of 50 μl of 0.3 M H_2SO_4 after 5 min of incubation with amplifier. The extent of reaction was carried out within the linear range of the detection method. The absorbance was read by an ELISA plate reader (BRL) at a wavelength of 490 nm.

Hirt DNA Isolation, Colony Hybridization, and Direct DNA Sequencing of PCR Fragments. The cells were harvested for vector DNA isolation by a modified alkaline lysis procedure (6), 24 h after transfection with the chimeric oligonucleotide. Hirt DNA was transformed into *Escherichia coli* DH5α cells (BRL). Colonies from Hirt DNA were screened for specific hybridization for each probe designed to distinguish the point mutation. Colonies were grown on ampicillin plates, lifted onto nitrocellulose filter paper in duplicates, and processed for colony hybridization. The blots were hybridized to ^{32}P-end-labeled oligonucleotide probes 711-A (5′-CCGCCTACAC-CCACTCG-3′) or 711-G (5′-CCGCCTACGCCCACTCG-3′) at 37°C in a solution containing 5× Denhardt's solution, 1% SDS, 2× SSC, and denatured salmon sperm DNA (100 μg/ml). Blots were washed at 52°C in TMAC solution (3.0 M teramethylammonium chloride/50 mM Tris·HCl, pH 8.0/2 mM EDTA/0.1% SDS). Plasmid DNA was made from 20 colonies shown to hybridize to either 711-G or 711-A, by using the Qiagen miniprep kit (Chatsworth, CA). Several hundred bases flanking position 711 of each plasmid was sequenced in both directions by automatic sequencing (ABI 373A, Applied Biosystems). A 192-bp PCR-amplified fragment was generated by Vent polymerase (New England Biolabs, MA), utilizing two primers (5′-CAATGTCCCTGATGTTATGCA-3′ and 5′-CGCTGGGCCAAGGACGCT-3′), corresponding to positions 630–650 and 803–822 of the alkaline phosphatase cDNA flanking position 711. The fragment was gel-purified and subjected to automatic DNA sequencing (ABI 373A, Applied Biosystems).

Oligonucleotide Stability Measurement. Ten nanograms of the ^{32}P-end-labeled oligonucleotide was mixed with 500 ng of the unlabeled oligonucleotide and transfected as described above. To reduce a nonspecific binding of oligonucleotides, cells were washed extensively with PBS and a solution containing 1 M NaCl and 0.56 M acetic acid (pH 2.5). A crude lysate was prepared by lysing the cells in a solution containing 10 mM Tris·HCl (pH 7.5), 0.5 mM $MgCl_2$, and 0.5% Triton

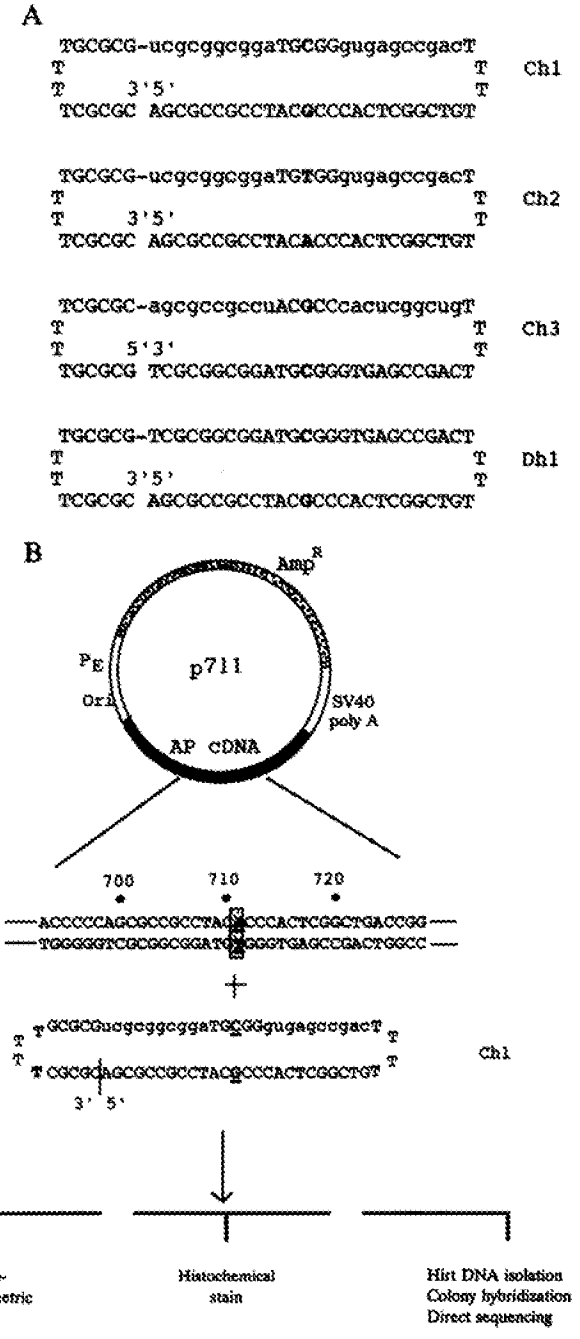

FIG. 1. (*A*) Sequences of chimeric oligonucleotides. Each RNA residue (in lowercase type) is modified by the inclusion of a 2′-O-methyl group on the ribose sugar. DNA residues are in uppercase type. (*B*) Strategy of episomal targeting. CHO cells are transfected with p711 containing a single point mutation, adenosine, at position 711 in the coding region of the alkaline phosphatase cDNA (solid box). The open box represents DNA containing the SV40 early promoter (P_E), SV40 origin of replication (ori), polyadenylylation addition site, and small intron sequence for splicing (SV40 poly A). The stippled box indicates the sequence from pBR322 encoding the origin of replication and β-lactamase (Amp^R) gene. The chimeric oligonucleotide Ch1 was introduced to CHO cells transfected with p711. The extent of the conversion to the wild-type phenotype was monitored at both biochemical and DNA sequence levels, by spectrophotometric measurement, histochemical staining, and analysis of Hirt DNA.

X-100 followed by phenol/chloroform extraction. Lysates were analyzed by 15% polyacrylamide gel containing 7 M urea followed by autoradiography. Oligonucleotides incubated in DMEM containing 10% FBS were processed and analyzed in the same manner.

RESULTS

Conversion of Inactive Alkaline Phosphatase to Active Enzymes by Chimeric Oligonucleotides. An expression plasmid containing the wild-type human liver/bone/kidney alkaline phosphatase cDNA under the control of the simian virus 40 (SV40) early promoter (18) is designated as pSV2Aalp', herein shortened to pHAP. An identical plasmid with the mutant version of the cDNA is designated pSV2Aalp' 711-A, herein shortened to p711. An illustrative example of the design of a chimeric oligonucleotide is diagrammed in Fig. 1*A*. Ten RNA residues flank either side of a 5-residue DNA stretch containing the base change desired for the correction of a defective gene. The chimeric oligonucleotide Ch1 (see Fig. 1*A*) was designed to correct the missense mutation to an active wild-type sequence. It has a guanosine residue at the site corresponding to the mutation. Another chimeric oligonucleotide, Ch2, has a design similar to Ch1 but with a single base change (G→A) at the site corresponding to position 711. The Ch3 molecule has the same sequence as Ch1 but the RNA and DNA sequences are inverted with the RNA stretches complementary to the noncoding strand of the alkaline phosphatase gene. The oligonucleotide Dh1 contains the same sequence as Ch1 but is composed entirely of DNA residues.

In our experimental design, various chimeric oligonucleotides were introduced into CHO cells previously transfected with p711, as shown in Fig. 1*B*. The extent of the conversion to the wild-type phenotype was monitored by histochemical staining, where red pigment was deposited on the cells expressing an active enzyme (19). When cells with the mutant gene were transfected with the chimeric oligonucleotide Ch1, red cells appeared at a high frequency (Fig. 2*A*). On the average, Ch1 promoted the conversion of approximately one in three transfected CHO cells from mutant to wild-type phenotype at 11 nM. In contrast, neither Ch2 nor Dh1 increased enzymatic activity (Fig. 2 *B* and *D*). The sequence of Ch2 is similar to that of the Ch1, except for the central adenosine substitution producing the identical sequence found in the plasmid p711. Thus, it would not form a mismatch at position 711. The frequency of conversion using Dh1, the DNA oligonucleotide with the sequence identical to Ch1, was also extremely low (Fig. 2*D*). This result confirmed the importance of the RNA sequence in the chimeric oligonucleotide. Upon transfection, plasmid p711 exhibited a trace of alkaline phosphatase activity because the 711 mutation does not inactivate enzymatic activity completely (18). The background enzymatic activity observed in cells treated with the plasmid p711 and Ch2 or Dh1 was similar to that of cells transfected with the p711 alone. Conversion to wild type was observed at a low level when cells were transfected with Ch3, a molecule in which the RNA stretches are complementary to the noncoding strand (Fig. 2*C*). It is not clear why there should be such a difference between the effectiveness of Ch1 vs. Ch3, but the transcribed strand may encounter a preferential treatment during the course of homologous pairing or DNA repair process. Transfection frequency measured by the expression of the wild-type plasmid pHAP was estimated to be 30% (Fig. 2*E*).

The enzymatic activity was also measured by a spectrophotometric method. A dose-dependent increase of alkaline phosphatase activity was observed up to 17 nM of Ch1 (Fig. 3). The enzymatic activity of cells treated with Ch1 at 17 nM was remarkably high, approaching 60% of that observed from cells transfected with the wild-type plasmid, pHAP. The increase was sequence-specific since the same amount of Ch1 did not affect the enzymatic activity of cells transfected with pHAP (Fig. 3). Furthermore, Ch2 chimeric oligonucleotide containing a single base-pair change from the Ch1 sequence did not show any increase in enzymatic activity. DNA oligonucleotide, Dh1, which contained the same sequence as Ch1 did not exhibit an increase. Thus, spectrophotometric measurements of alkaline phosphatase activity were consistent with the result from the histochemical staining.

Correction of a Point Mutation of the Targeted DNA Sequence by the Chimeric Oligonucleotides. To confirm the change at the DNA sequence level, a Hirt extract was made from the cells transfected with the p711 and various oligonucleotides by a modified alkaline lysis procedure (6), 24 h after transfection with the chimeric oligonucleotide. Hirt DNA transformed DH5α cells efficiently, resulting in 10^4 ampicillin-resistant colonies from 10^6 transfected CHO cells. DH5α transformants were screened for specific hybridization with a probe designed to distinguish between the point mutation (underlined A) and the wild-type (underlined G) sequence (711-A, 5'-CCGCCTACACCCACTCG-3' and 711-G, 5'-CCGCCTACGCCCACTCG-3'), corresponding to positions 703–719 of mutant and normal cDNAs, respectively (18). A representative autoradiogram of duplicate filters hybridized to the 711-G or 711-A probe from each plate is shown in Fig. 4 *A* and *B*. The frequency of correction was measured by averaging the number of colonies hybridized to the 711-G or 711-A probe by using more than 400 colonies on the multiple plates generated from at least two transfection experiments (Table 1). Similar frequency of conversion was observed between two batches of Ch1 prepared by separate synthesis. Approximately 70% of the colonies generated from the Hirt DNA made from cells transfected with p711 and Ch1 hybrid-

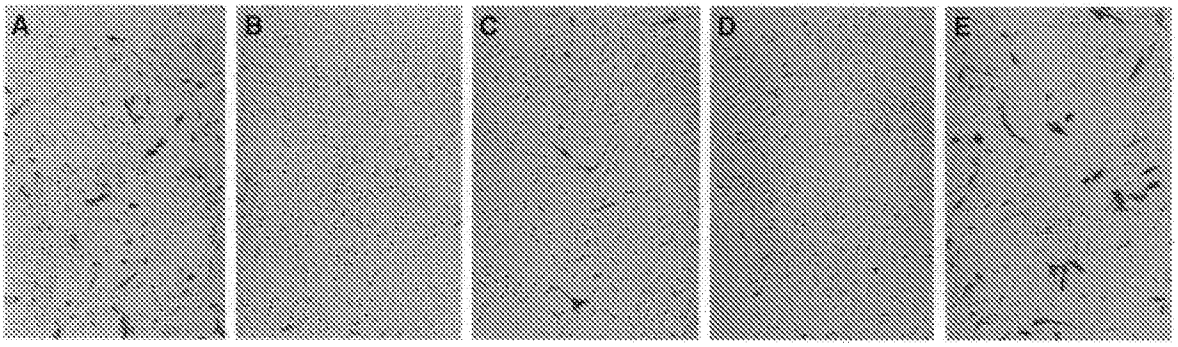

FIG. 2. Histochemical staining of alkaline phosphatase activities of CHO cells transfected with various oligonucleotides. (*A–D*) Histochemical staining of CHO cells transfected with 10 μg of p711 followed by various oligonucleotides at 11 nM, Ch1, Ch2, Ch3, or Dh1, respectively. (*E*) CHO cells transfected with 10 μg of pHAP and 11 nM Ch1. The red-stained cells represent CHO cells expressing an active alkaline phosphatase due to an A → G conversion by chimeric oligonucleotides. Unstained cells represent either untransfected CHO cells or CHO cells containing p711 that is not converted. Each micrograph shows CHO cells in a 6-well plate and contains >600 cells per field. (×4.)

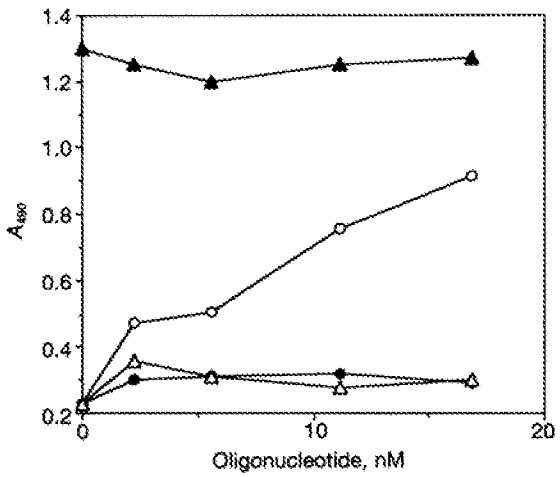

FIG. 3. Dose-dependent alkaline phosphatase activity. Enzymatic activity of CHO cells transfected with p711 and Ch1 (open circles), Ch2 (solid circles), or Dh1 (open triangles) oligonucleotides. The solid triangle represents the enzymatic activity measured from CHO cells transfected with pHAP and Ch1. The absorbance represents an average of six absorbance readings obtained from the duplicate assays of three transfection experiments within 10% of standard deviation.

ized to the 711-A probe, while 30% of colonies exhibited hybridization to the 711-G probe (Table 1). Thus, a correction frequency of 30% was reproducibly observed at 11 nM Ch1. Hybridization was specific and no cross-hybridization was observed between the two populations. DNA sequencing was carried out with plasmid DNAs prepared from 20 of these colonies in both directions utilizing two primers (5′-CAATGTCCCTGATGTTATGCA-3′ and 5′-CGCTGGGC-CAAGGACGCT-3′), corresponding to positions 630–650 and 803–822 of the alkaline phosphatase cDNA flanking position 711. The sequence conversion was confirmed in each case and no other alteration in sequence was observed within several hundred bases surrounding the target nucleotide (data not shown). All colonies from the Hirt extract prepared from 20 colonies of the Ch2- to Dh1-treated cells hybridized to the 711-A probe only (Table 1). Some colonies from the Hirt extract of the Ch3 hybridized to the wild-type probe but to a much lesser extent than that of the Ch1 (Table 1). These results confirmed that the differential alkaline phosphatase activities exhibited were due to the correction of the point mutation (A → G) at the DNA sequence level.

RecA-deficient *E. coli* strains used to propagate plasmid DNA are capable of repair and homologous pairing functions by using episomal DNA (21). To rule out the possibility that the sequence conversion is mediated by *E. coli*, direct DNA sequencing of a PCR-amplified fragment of Hirt DNA was carried out. Two primers flanking position 711 were utilized to generate a 192-bp fragment through the action of Vent polymerase. The results indicated that position 711 was a mixture of adenosine (70%) and guanosine (30%) when the Hirt DNA sample was made from the cells transfected with the combination of p711 and Ch1 (Fig. 4*C*). In contrast, no mixed sequence was observed at position 711 when Hirt DNA was made from oligonucleotide Dh1 (Fig. 4*D*). These results established clearly that sequence correction by the chimeric oligonucleotide occurred in mammalian cells.

Stability of Chimeric Oligonucleotides. The stability of the chimeric oligonucleotide was measured intracellularly and in growth medium containing 10% FBS. Ten nanograms of radiolabeled oligonucleotide, Ch1, was added to the same transfection experiment in which histochemical staining and Hirt DNA analyses was conducted. The chimeric oligonucleotides are extremely stable. No detectable degradation was observed when chimeric oligonucleotide was incubated in growth medium containing 10% FBS, after a 24-h incubation (Fig. 5, lane 3). Moreover, oligonucleotide isolated from cells did not exhibit any degradation during the same incubation time (Fig. 5, lane 2). Only monomers of the chimeric oligonucleotide were detected when isolated from cells 24 h after incubation. Thus, under the experimental conditions employed here, no end-to-end ligation of chimeric oligonucleotides was observed.

DISCUSSION

These results demonstrate that it is possible to correct a mutant base in a gene by using an oligonucleotide-based strategy with relatively high frequency in a sequence-specific manner. A major modification of the oligonucleotide, incorporation of RNA residues complementary through the central region of

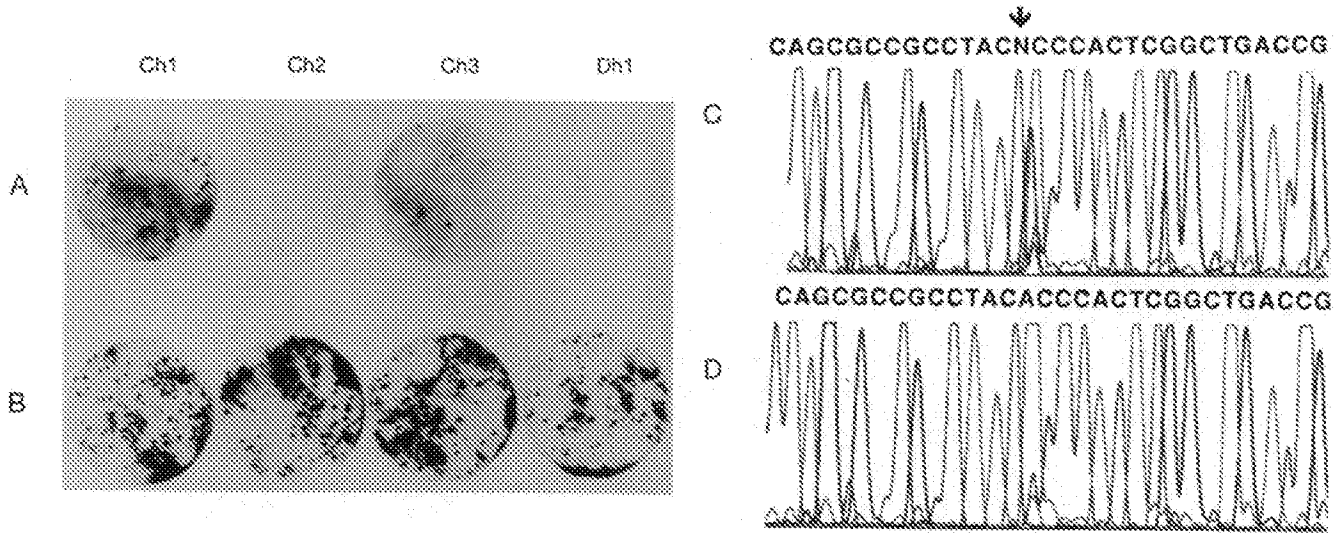

FIG. 4. Analysis of Hirt-extracted DNA from CHO cells transfected with plasmid p711 and oligonucleotides Ch1, Ch2, Ch3, or Dh1. (*A*) Hybridization pattern of colonies transformed by Hirt DNA from CHO cells transfected with plasmid p711 and oligonucleotides Ch1, Ch2, Ch3, or Dh1 with ^{32}P-end-labeled oligonucleotide probe 711-G. (*B*) Duplicate nitrocellulose filter hybridization pattern with ^{32}P-end-labeled oligonucleotide probe 711-A. (*C* and *D*) Direct sequencing of the 192-bp PCR-amplified fragment from Hirt DNA from CHO cells transfected with p711 plasmid followed by the Ch1 or Dh1, respectively. The 192-bp PCR-amplified fragment was generated. Arrow indicates position 711.

Table 1. Hybridization pattern of transformants from Hirt extract prepared from duplicate transfections of the p711 plasmid and various oligonucleotides at 11 nM

Oligo-nucleotide	No. of transfections	Total no. of colonies per plate	No. of colonies hybridizing to 711-G	No. of colonies hybridizing to 711-A	% conversion
Ch1	1	84	32	54	38
		189	70	117	37
		219	74	143	34
	2	139	42	98	30
		162	49	110	30
		159	51	108	32
Ch2	1	108	0	108	0
		90	0	90	0
	2	218	0	218	0
		148	0	148	0
Ch3	1	190	3	185	2
		151	4	145	3
	2	189	0	185	0
		143	0	143	0
Dh1	1	217	0	217	0
		180	0	180	0
	2	157	0	157	0
		188	0	188	0

the targeted strand, appears necessary to make this approach feasible. The importance of RNA in the present study was demonstrated by the differential activities of oligonucleotides containing identical sequence with or without RNA stretches, Ch1 and Dh1, in eliciting the genetic change detected at both the biochemical (Figs. 2 and 3) and genetic (Fig. 4 and Table 1) levels. We hypothesize that the perturbation and partial destacking of the RNA·DNA base pairing in the chimeric oligonucleotide facilitates the initiation of homologous alignment and influences the rate of the recombination. Several enzymes that facilitate homologous pairing *in vitro*, *E. coli* RecA and *U. maydis* Rec2 proteins, have been found to bind preferentially to the RNA·DNA chimeric oligonucleotide (unpublished observation). Once the oligonucleotide is brought into complementary register with the target, the mismatch base pair is processed by a series of repair activities that are not fully elucidated.

It is probable that the gene correction event is mediated by a specific mismatch repair system because Ch1 and Ch3 were the only effective chimeric molecules in enabling the changes. Both Ch1 and Ch3 are designed to form the mismatched base pair with the target gene, whereas Ch2 forms a perfect complementary match. Highly efficient repair activity has been observed in nuclear extracts prepared from several mammalian cell lines (22–24). Such processes are often strand-specific and bidirectional relative to a nick that is located 5′ or 3′ of the mismatch (22, 24). It is possible that RNA·DNA chimeric oligonucleotides may not only enhance the pairing activity but also facilitate the mismatch repair activity to the targeted strand preferentially. The higher rate of correction exhibited by Ch1 over Ch3 suggests a correlation between DNA repair and transcription, identifying an important strategy for designing effective chimeric oligonucleotides. Similar correlations of transcription and homologous recombination (25, 26) and DNA repair (27, 28) have been established in other systems.

Extrachromosomal homologous recombination has been reported to be more frequent than chromosomal recombination, albeit lower than what we observed in this study (29–33). Relatively high frequency approaching 15% was observed when an episomal DNA and a sequence-specific endonuclease were injected into the *Xenopus laevis* oocyte nucleus (31). When uncleaved episomal DNA was injected to nucleus, no recombination event was detected (30, 31). In another case, cotransfection of an uncleaved DNA and a high concentration of double-stranded oligonucleotides (molar ratio of oligonucleotide to plasmid of 143) in mammalian cells did not enable detectable homologous recombination event (29). Thus, a cleavage of episomal DNA was required for efficient recombination to occur in both systems, suggesting a nonconservative single-strand annealing mechanism for extrachromosomal homologous recombination (29–33). In our experiments, a high frequency of correction was observed when uncleaved extrachromosomal DNA was introduced to mammalian cells followed by 11 nM chimeric oligonucleotides (molar ratio of the oligonucleotide to plasmid = 7). Expression of one corrected plasmid per cell may result in conversion of red cells as seen in histochemical staining (Fig. 2). However, DNA sequence analysis by selective hybridization suggests that the frequency of episomal targeted mutagenesis approaches 30% (Table 1). Moreover, a mixture of adenosine (70%) and guanosine (30%) was observed at position 711 by direct sequencing of the PCR-amplified fragment from the Hirt DNA (Fig. 4C). This result indicates a correction efficiency of 30% among the total population of episomal DNA with no prior biased selection, consistent with the selective hybridization results. Thus, chimeric oligonucleotides appear to facilitate cellular recombination and repair processes of episomal DNA at a frequency higher than previous studies (29–33).

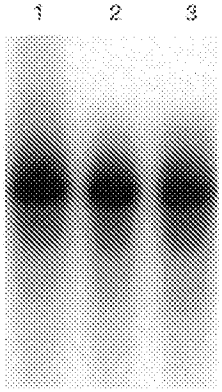

FIG. 5. Stability of chimeric oligonucleotide. Autoradiogram of ^{32}P-end-labeled chimeric oligonucleotide Ch1 (lane 1) and Ch1 extracted from CHO cells and medium (lanes 2 and 3), 24 h after transfection.

In mammalian cells, homologous targeting occurs against a background of nonhomologous events that are more common. Ingenious procedures have been devised to select or screen for the rare successful targeting products, but low absolute frequency of specificity remains a serious limitation (1–5). In this work, a high frequency of the targeted event was detected without selection. We considered the possibility that the chimeric oligonucleotide causes nonhomologous recombination events leading to spurious mutations or random integration. We have found no evidence of either type since DNA sequencing of several hundred bases surrounding the target base revealed no alteration in sequence. The chimeric oligonucleotide was stable and remained as a monomer inside the cells, indicating that these molecules were not ligated into concatamers (Fig. 5, lane 2). This observation also argues against the participation of chimeric oligonucleotide in nonhomologous recombination, in which end-to-end ligation of the double-stranded DNA is often observed prior to integration (see ref. 5 and references therein). Moreover, the kinetics of conversion by chimeric oligonucleotides appears to be fast (<24 h), while most integration events of the double-stranded DNA occur over much longer time periods (1–5). To address random integration of Ch1, a Southern blot analysis was carried out. The chimeric oligonucleotide hybridized to a single *Sau*3A fragment containing position 711 of Hirt DNA and did not hybridize at all to the CHO genomic DNA (data not shown). Thus, within our detection limit, we conclude that targeted conversion by this oligonucleotide is highly sequence-specific and more frequent than random nonhomologous recombination events.

This study outlines a way to expand the repertoire of target sequences and to improve the specificity of the targeting event. Viral genes are potential targets for chimeric oligonucleotides as strategically placed mutations can be introduced into the episomal genome to inactivate key viral proteins. Recently, we have achieved a high frequency of genomic targeting by the use of the chimeric oligonucleotide without evidence of random integration or mutation. Such observations should extend the applicability of this therapeutic strategy to human genetic diseases.

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